PB86-129335

Hazard Evaluation Division, Standard Evaluation Procedure: Acute Toxicity Test for Estuarine and Marine Organisms (Mollusc 48-Hour Embryo Larvae Study)

(U.S.) Environmental Protection Agency
Washington, DC

Jun 85

U.S. Department of Commerce
National Technical Information Service
NTIS

PB86-129335

EPA-540/9-85-012
June 1985

HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

ACUTE TOXICITY TEST FOR ESTUARINE AND MARINE ORGANISMS

(Mollusc 48-Hour Embryo Larvae Study)

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REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

50272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO.	2.	3. Recipient's Accession No. PB86 129335/AS

4. Title and Subtitle
HAZARD EVALUATION DIVISION, STANDARD EVALUATION PROCEDURE
Acute Toxicity Test for Estuarine and Marine Organisms (Mollusc 48-Hour Embryo Larvae Study)

5. Report Date
June 1985

6. EPA-540/9-85-012

7. Author(s)
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8. Performing Organization Rept. No.

9. Performing Organization Name and Address
U.S. Environmental Protection Agency/OPP/HED/(TS-769C)
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Washington, D.C. 20460

10. Project/Task/Work Unit No.

11. Contract(C) or Grant(G) No.
(C)
(G)

12. Sponsoring Organization Name and Address
Same as #9.

13. Type of Report & Period Covered

14.

15. Supplementary Notes
Supplement to Pesticide Assessment Guidelines Subdivision E - Hazard Evaluation: Wildlife and Aquatic Organisms (EPA-540/9-82-024 and NTIS Order Number PB83-153908), October, 1982.

16. Abstract (Limit: 200 words)

The Standard Evaluation Procedure (SEP) for the Mollusc 48-Hour Embryo-Larvae Toxicity Test is a guidance document primarily intended for Agency reviewers and the regulated industry who evaluate ecological effects data specified in 40 CFR Part 158.145. The SEP is also intended to provide information to the general public indicating how the Agency evaluates these types of studies. As such, it is designed to supplement Subdivision E of the Pesticide Assessment Guidelines: Hazard Evaluation - Wildlife and Aquatic Organisms. This SEP provides an Introduction, Materials and Methods, Reporting Requirements, Reviewer Evaluation, and Appendix of appropriate methods to guide the review and scientific evaluation of pesticide effects on estuarine/marine fish.

The SEP for the Estuarine Fish 96-Hour Acute Toxicity Test is only one of a number of SEP's published by the National Technical Information Service as a supplement to Subdivision E of the Pesticide Assessment Guidelines.

17. Document Analysis a. Descriptors

b. Identifiers/Open-Ended Terms

c. COSATI Field/Group

18. Availability Statement	19. Security Class (This Report)	21. No. of Pages
Unclassified and freely available.	Unclassified.	17
	20. Security Class (This Page) Unclassified.	22. Price

STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.

John W. Melone, Director
Hazard Evaluation Division

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ACUTE TOXICITY TEST FOR ESTUARINE AND MARINE ORGANISMS: MOLLUSC 48-HOUR EMBRYO LARVAE STUDY

I. INTRODUCTION

A. When Required

Acute toxicity studies with mollusc are required to support registration of an end-use product intended for direct application to the estuarine or marine environment. This study is also necessary when it is expected that the pesticide would enter this environment in significant concentrations because of its expected use or mobility.

B. Purpose

- To establish acute toxicity levels of the active ingredient to nontarget marine and estuarine organisms;
- To compare toxicity information with measured or estimated pesticide residues in the estuarine or marine environment to assess potential impact to invertebrates;
- To provide support for precautionary label statements to minimize adverse effects to estuarine or marine non-target organisms; and
- To indicate the need for further testing and/or field studies.

C. Test Material

1. Technical Grade

Tests must be conducted with the technical grade of the active ingredient. If more than one active ingredient constitutes a technical product then the technical grade of each active ingredient must be tested separately.

2. End-Use Product

The applicant may be required to test the end-use product as well if:

- The end-use product will be introduced directly into the marine or estuarine environment when used as directed;
- The mollusc EC_{50} of the technical grade of the active ingredient is equal to or less than the expected environmental concentration in the marine or estuarine environment when the end-use product is used as directed;

- An ingredient of the end-use product is expected to enhance the toxicity of the end-use product beyond that expected from the active ingredient(s); or

- The technical product is insoluble in water but the formulated product is soluble in water. In that case the carriers or inerts of the formulated product must be tested in a solvent control.

II. MATERIALS AND METHODS: TESTING STANDARDS/RECOMMENDATIONS

A. Acceptable Protocols

EEB does not endorse any one protocol. It is sometimes necessary and desirable to alter the procedures presented in published protocols to meet the needs of the chemical or test organisms used. However, EEB does recommend some protocols as guidance for developing mollusc acute toxicity tests. These protocols include:

American Public Health Association, American Water Works Association and Water Pollution Control Federation. 1981. Standard Methods for the Examination of Water and Wastewater. Fifteenth edition. Publication office: American Public Health Association, 1015 18th Street NW, Washington, DC 20036.

American Society Testing Materials. 1980. Standard Practice for Conducting Static Acute Toxicity Tests with Larvae of Four Species of Bivalve Molluscs. E 724-80, Published by the ASTM Committee on Standards, 1916 Race Street, Philadelphia, PA, 19103.

Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/375-009. 61 pp.

Woelke, C.E. 1967. "Measurement of Water Quality with the Pacific Oyster Embryo Bioassay." Water Quality Criteria, ASTM STP 416, p. 112.

These referenced protocols are presented as flexible guidance to help researchers design scientific protocol and to help the reviewer validate studies. It is important to recognize that mollusc tests are validated based on whether they provide scientifically sound information on the acute toxicity of the test material to mollusc that is useful in risk assessments and whether they fulfill guideline requirements. This is more important than whether they follow a referenced protocol step-by-step.

B. Test Organisms

1. Acceptable Species

The selected species should have a demonstrated sensitivity to known toxicants. If possible they should be species that occur in the area of exposure or that represent exposed species.

Numerous species are specifically referenced in "Methods of Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians" (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975, and ASTM E 729-80). The following species are preferred because there is a substantial data base available on them:

Crassostrea giga	Pacific oyster
Crassostrea virginica	Eastern oyster
Mytilus edulis	Mussel
Mercenaria mercenaria	Quahog

Some other species may be acceptable if they have been shown to be sensitive in acute toxicity tests.

2. Size/Age/Physical Condition

Embryos should be tested within one hour of spawning and after fertilization. The eggs are fertilized as they are discharged from the female; they are then added to treated test solution.

The adults must be from clean water that is not subject to point or non-point pollution so they will not have developed genetic resistance to toxicants that would make their offspring less sensitive to toxicants.

3. Source/Acclimation

Mollusc larvae are obtained from adult brood stock in the laboratory. This brood stock is obtained either from the wild or from commercial sources and caused to spawn. The referenced protocols provide procedures for causing molluscs to spawn.

All organisms must be from the same source. This may include laboratory or commercial stocks. Organisms captured in the wild are acceptable provided they meet the requirements pertaining to physical condition and age/size criteria mentioned above.

C. Test Solution

1. Source of Dilution Water

An adequate supply of dilution water that meets the minimum requirements must be available. The water may be natural or reconstituted but must be able to support the test animals without stress. Specifically, control larvae spawned by adults conditioned in the dilution water must not incur more than 10% abnormal development or 30% mortality in 48 hours.

Natural or reconstituted seawater of 30 to 34% salinity and pH of 8 to 8.3 should be used when testing marine (stenohaline) mollusc, and 10 to 17% salinity and pH 7.7 to 8.0 with estuarine (euryhaline) mollusc species. Natural seawater is considered to be of constant quality if the weekly range of salinity is less than 6% and if the monthly pH range is less than 0.8 of a pH unit. See the protocols by the American Society for Testing Materials (1980) or the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975) for guidance on specific amounts of minerals in reconstituted seawater. Commercial sources of seawater mixture are acceptable provided they do not adversely affect the test organism or alter the toxicity of the test material.

2. Temperature

Most protocols recommend 20°C as the temperature. However it is possible that a higher temperature (e.g., 25°C) may be acceptable when the adult molluscs were collected from warmer waters as in southern areas. Temperature should not vary more than 2°C during the test. Laboratories using molluscs collected in the south where ambient temperatures may already be 27-28°C may have difficulty raising the temperature the 5-10°C necessary to induce spawning without exceeding the lethal limit (33-34°C) for adult molluscs.

D. Testing System

1. Test Vessels

Glass 1 Liter beakers are preferred. It is strongly recommended that each treatment be tested in duplicate or even triplicate. There should be 20,000 to 30,000 fertilized eggs per liter of test solution.

2. Photoperiod

A 16-hour light and an 8-hour dark photoperiod should be provided, with a 15 to 30 minute transition period between light and dark.

3. Loading

There should be 20,000 to 30,000 fertilized eggs per liter of test solution.

4. Solvents

If solvents other than water are necessary, they should be used sparingly, not to exceed 0.5 ml/L in any test solution. The following solvents are preferred:

dimethyl formamide
triethylene glycol
methanol
acetone
ethanol

5. Aeration

Aeration is not recommended because it could cause volatilization of test material. However if aeration is necessary, the residues in the test solution must be measured at the beginning and near the end of the study.

E. Test Design

1. Test Levels (Nominal/Measured)

Initially, range finding tests may be necessary to define concentrations of the toxicant needed for definitive studies. Test reports should provide information describing range finding study procedures and results. The information should include sample sizes, dosage levels, and mortality data. If there is a problem with solubility or volatibility, if the test containers are aerated, or if it is a flow-through test, the residue levels must be measured at the beginning and at the end of the test period.

If it can be shown that a chemical will have an EC_{50} greater than 100 ppm, a definitive study need not be performed.

Definitive acute toxicity tests normally are designed to include one or more control groups and a geometric series of at least five toxicant concentrations to be tested. Each designated treatment group should be exposed to a concentration of toxicant that is at least 60% of the next highest concentration. If a formulated product is tested, it should be clearly stated in the test report whether results are expressed in terms of active ingredient alone or as total formulated product.

2. Number of Test Animals

Numbers of mollusc larvae per level are determined as a certain density per volume of test solution. If oyster larvae are used, normally, 20,000 to 30,000 are tested per liter. Replicate test chambers may be used, but the number of organisms would still be measured as number of larvae per volume of test solution.

3. Controls

Each test requires a concurrent control using the same dilution water and same concentrations of larvae at each test level. The number of control replicates should be four or the equivalent of 10% of the total number of treatment replicates, whichever is greater. If any solvent other than water is used a solvent control should be conducted. This solvent control should contain the highest concentration of the solvent that was added to any of the test solutions. A test is not acceptable if there is more than 30% mortality in the control. If mean mortality exceeds 2% in controls, each treatment response should be corrected for the mean level of control using the following equation:

$$\text{Net Percent Mortality For Each Level} = \left(\frac{\text{percent of surviving larvae for each treatment replicate}}{\text{mean percent of surviving control larvae per control replicate}} \right) \times 100$$

4. Beginning the Test

Fill each test container with the same volume of test solution and toxicant. The larvae are then mixed with the test solution at approximately 20,000 to 30,000 per liter.

5. Measurement of Diluent Characteristics

Temperature should be recorded hourly throughout the acclimation and test periods in at least one test chamber if the test containers are not in a temperature controlled water bath because air temperature may change more frequently and to a greater extent than water, thus affecting the test container temperature. If the temperature is controlled by a water bath, the temperature of the bath may be recorded every six hours. The range and average temperature must be recorded.

The dissolved oxygen (DO) concentration must be measured at the beginning of the test and at the end of the test in the control and the high, medium, and low concentration. The DO level must be between 60% and 100% of saturation.

The pH should be measured at the beginning and end of the test in the control and the high, medium, and low toxicant concentrations.

6. Chemical Analysis

It may be necessary to chemically analyze test solutions to determine exact concentrations of pesticides. It is particularly important that residues are measured if:

- The test solutions were aerated;
- The test material was volatile, insoluble, or precipitated out of solution;

- The test containers were not made of glass or stainless steel;
- The test chemical is known to adsorb to the test container's structural material; or
- A flow-through system is used (measurement verifies accuracy of metering system).

III. REPORTING REQUIREMENTS

The test report submitted to the Agency must fully describe the materials and methods used to perform the study. The reviewer must be able to determine from the report that the study was performed under conditions that render the results acceptable for use in a risk assessment and/or for fulfilling a guideline requirement. The following information is particularly important for a complete evaluation.

A. Test Material

If the study is to be performed with the technical grade product, the test material should be clearly identified as to source, batch, and exact purity. Simply identifying the material as technical may not be sufficient because the percent active ingredient of some newer products may increase with time as the manufacturing process is improved to produce greater purity.

For studies involving the end-use product, the exact percent of the active ingredient and the type of formulation (e.g., granular, wettable powder) of the test material should be described. It should clearly state in the test report whether the results are expressed in terms of active ingredient or as formulation.

B. Dilution Water

Test reports submitted to the Agency should include a complete description of dilution waters used in the test. Descriptions should include identification of the source, the chemical characteristics of the water, and information on any pretreatment.

C. Holding of Test Organisms

Test reports should include complete information on holding and acclimation conditions including feeding schedules and treatment for diseases.

D. Observable Effects Criteria

According to Woelke, fertilized eggs develop into free-swimming, fully shelled veliger larvae in the 48 hours covered by this protocol. He assumes that failure to do so will break the life cycle of, in this case, the Pacific oyster.

The measured response in this test should be the percentage of larvae dying or failing to develop complete shells. Larvae possessing misshapen shells or otherwise malformed shells are considered normal (survivable) as long as the shell is complete. Incidences of misshapen shells or otherwise malformed shells should be reported.

To determine percent response per level, samples containing about 150 to 250 larvae taken from each culture are examined. The number of fully developed larvae compared to not fully developed larvae are counted and used in the following equation to obtain percent response per level.

$$\text{Percent reduction per test level} = \left(\frac{\text{\# fully dev. in control} - \text{\# fully dev. in test culture}}{\text{\# fully dev. in control culture}} \right) \times 100$$

E. Calculated EC50

The statistically calculated EC_{50} with 95% confidence limits and the method of calculation must be presented. In lieu of a calculated EC_{50} the study may show that the EC_{50} is greater than 100 ppm. The mean percent abnormal larvae from each test level should be plotted on probability paper, with the concentrations converted to logs. The EC_{50} may then be calculated by linear regression.

F. Temperature/DO/pH

Dissolved oxygen, pH measurements and the range and average temperature during the study should be reported.

G. Chemical Analysis

The test report should provide information on the methods (references) utilized and the results of analyses. Residues found at the beginning and end of the study should be reported.

H. Testing Protocols

The test report should include reference to the testing protocol(s) used during the study.

IV. REVIEWER'S EVALUATION

A. Review of Test Conditions

The reviewer should identify each aspect of the reported procedure that is inconsistent with recommended protocol. The significance of these deviations must be determined. The number of deviations and their severity will determine the validity of the study and the interpretation of the results.

B. Verification of Statistical Analysis

The reviewer should ensure that the EC_{50} has been properly derived by recalculating these values. An acceptable acute toxicity test should provide not only an EC_{50} but also a NOEL (no observed effect level) and a slope of the dose/effect response. The NOEL is the highest test level at which no effects were observed.

If the recalculated results differ substantially from the submitted results, the reviewer should note this and attempt to explain the differences.

C. Conclusions

1. Categorization of Results

The significance of inconsistencies in the test procedures must be determined by the reviewer so that the results of the test can be categorized as to whether they fulfill Part 158 regulations and are useful in performing a risk assessment. Categories are described as:

- Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent, however the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

- Supplemental: Studies in this category are scientifically sound, however, they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements, however, the information may be useful in a risk assessment. Some of the conditions that may place a study in a supplemental category include:

  - Unacceptable test species;
  - Inappropriate test material;
  - Concentrations tested were less than 100 ppm but not high enough to produce an effect on the organisms or a precise EC_{50}; and

- Deviations from recommended test solution characteristics (variations in DO, temperature, hardness, and pH can affect toxicological response).

° Invalid: These studies provide no useful information. They may be scientifically unsound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment.

Examples of studies placed in this category commonly include those where the test system was aerated, test vessels were constructed from materials other than glass, or there were problems of solubility or volatility of the test material. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that test organisms were actually exposed to nominally designated concentrations.

A study where the test material was not properly identified can also be made invalid.

2. Rationale

Identify what makes the study supplemental or invalid. While all deviations from recommended protocol should be noted, the reviewer is expected to exercise judgment in the area of study categorization.

3. Repairability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data on the study.

D. Descriptive Classification

The reviewer should indicate what the results were and how much information can be drawn from them. At a minimum, an acute toxicity test will provide an EC_{50} with 95% confidence limits. This should allow classifying the test material based on the following scheme:

EC_{50} (ppm)	Category Description
< 0.1	very highly toxic
0.1 - 1	highly toxic
1 - 10	moderately toxic
10 - 100	slightly toxic
> 100	practically non-toxic

These descriptive categories are for inter-chemical comparison only and do not reflect actual environmental hazard to the test organism. The results may provide other useful information such as slope or a no observed effect level (NOEL). These additional data are useful in a risk assessment.

E. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other branches.